Biomechanical and Rheological Characterization of Mild Intervertebral Disc Degeneration in a Large Animal Model

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ABSTRACT: Biomechanical properties of healthy and degenerated nucleus pulposus (NP) are thought to be important for future regenerative strategies for intervertebral disc (IVD) repair. However, which properties are pivotal as design criteria when developing NP replacement materials is ill understood. Therefore, we determined and compared segmental biomechanics and NP viscoelastic properties in normal and mildly degenerated discs. In eight goats, three lumbar IVDs were chemically degenerated using chondroitinase ABC (CABC), confirmed with radiography and MRI after euthanasia 12 weeks post-operative. Neutral zone (NZ) stiffness and range of motion (ROM) were determined sagitally, laterally, and rotationally for each spinal motion segment (SMS) using a mechanical testing device. NPs were isolated for oscillatory shear experiments; elastic and viscous shear moduli followed from the ratio between shear stress and strain. Water content was quantified by weighing before and after freeze-drying. Disc height on radiographs and signal intensity on MRI decreased (6% and 22%, respectively, $p < 0.01$) after CABC treatment, confirming that chemical degeneration provides a good model of disc degeneration. Furthermore, CABC-injected IVDs had significantly lower NZ stiffness and larger ROM in lateral bending (LB) and axial rotation (AR) than controls. Rheometry consistently revealed significantly lower (10–12%) viscoelastic moduli after mild degeneration within goats, though the inter-animal differences were relatively large (complex modulus ~12 to 41 kPa). Relative water content in the NP was unaffected by CABC, remaining at ~75%. These observations suggest that viscoelastic properties have a marginal influence on mechanical behavior of the whole SMS. Therefore, when developing replacement materials the focus should be on other design criteria, such as biochemical cues and swelling pressure. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:703–709, 2013

Keywords: nucleus pulposus; biomechanics; viscoelasticity; animal model; disc degeneration

Low back pain is attributable to multiple biological, psychological, and socioeconomic factors, among which intervertebral disc (IVD) degeneration plays an important etiological role.[1–3] As degeneration appears to start with changes in the nucleus pulposus (NP),[4] several research groups have been working on strategies for NP regeneration.[5–9] Different cell types have been identified as potential therapeutic candidates for disc regeneration, including chondrocytes, NP cells, and mesenchymal stem cells (MSCs).[10,11] The behavior of these regenerative cells is governed by biochemical (pH, oxygen, and glucose levels)[12,13] and mechanical stimuli.[14] More specifically, these react differently to varying degrees of stiffness of their substrates, in terms of differentiation,[15–17] proliferation and migration,[18] and matrix production.[19] Therefore, regeneration strategies often aim to mimic the specific environment of the native, healthy NP tissue by introducing replacement materials combined with regenerative cells. However, the conditions in the degenerated IVD are also relevant, as they represent the environment that will influence the capacity of newly introduced cells.

A defining factor of this environment is the viscoelasticity of the surrounding matrix, measured as the mechanical response to dynamic shear load. These properties have only recently been marginally addressed in cadaveric human[20] and animal[21] studies on NP material, but not yet in a reproducible disc degeneration model. Furthermore, bending and torsion loading of a whole spinal motion segment (SMS) markedly influences the IVD. Therefore, we determined biomechanical characteristics of the whole SMS and viscoelastic properties of the NP in healthy and mildly degenerated discs. We used a goat model of chemically induced mild IVD degeneration that was established in earlier studies by our group.[22,23]

MATERIALS AND METHODS

Animals and Surgical Procedure

Eight skeletally mature female Dutch milk goats (age 3–4 years, weight 92 ± 10 kg) were obtained from a local farmer. The research protocol was approved by the Scientific Board and the Animal Ethics Committee of the VU University Medical Center in Amsterdam. To establish mild degeneration, three out of six lumbar IVDs in each goat were injected with the enzyme chondroitinase ABC (CABC), which cleaves proteoglycans, thereby providing the onset of the degenerative cascade. Goats were operated using the same procedure as previously described.[22,23] Briefly, lumbar IVDs were approached through a dorsal left paravertebral incision and exposed after mobilization of the psoas muscle. CABC (Sigma–Aldrich, Inc., St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and diluted to a concentration of 0.25 U/ml. After identifying the position with an image

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intensifier, three lumbar IVDs were randomly injected with a 29G needle, leaving three levels as negative controls. A mean of one hundred thirty microliters (range 80–200 μl) of CABC-solution was delivered into each NP under manual pressure guidance. After suturing and recovery, animals were allowed to move freely in their cages and were fed ad libitum. Twelve weeks postoperatively the animals were euthanized with an overdose of sodium pentobarbital, after which the lumbar spines were harvested en-bloc from T13 to L6. To confirm that mild degeneration had occurred, radiographic and MRI analyses were conducted.

Radiological Analysis

Before surgery and before autopsy, conventional lateral radiographs of the lumbar spine were obtained. To adjust for inter-animal differences and projection errors on the radiographs, a disc height index (DHI) was calculated as previously described.[22] In short, disc and vertebral body height were derived by averaging three measurements of each IVD and vertebral body height. Subsequently, relative disc height was calculated as the ratio between average IVD height and average caudal vertebral height. Finally, the relative disc heights on radiographs before autopsy were expressed as a percentage of the pre-operative disc heights [DHI = preautopsy DHI/preoperative DHI × 100%]. MRI scans were taken in sagittal direction, using a T2 weighted sequence and slice thickness of 2 mm on a 1.5 Tesla Siemens Symphony machine. MRI scans were analyzed using the MRI index.[24] In short, the images were transferred to commercially available image processing software (OsiriX Imaging Software, www.osirix-viewer.com) where the NP was identified manually as region of interest. Surface area and signal intensity were then computed automatically, and their product resulted in an MRI index for each IVD. To compare among MRI scans, values were expressed as ratios of a reference tube filled with demineralized water, which was scanned together with the goat spines (Fig. 1).

Biomechanical Testing

Biomechanical testing was performed using a mechanical testing device with a maximum capacity of four adjacent motion segments. To prepare specimens for testing, either T13 to L4 or L1 to L5 were isolated en-bloc after which the posterior and transverse processes were removed. Each spinal sample thus contained two CABC-injected and two healthy IVDs, and mechanical testing was carried out as described previously.[25,26] In short, the two outer vertebral bodies were embedded in a low melting point bismuth alloy, and the lumbar spine was placed in a mechanical testing device (Instron 8872, Canton, MA). Markers containing light-emitting diodes (LEDs) were positioned on each vertebral body, and motions of the LEDs were recorded by an optoelectronic 3D movement registration system (Optotrak 3020, Northern Digital, Inc., Waterloo, ON, Canada). The lumbar spine was positioned in its neutral, horizontal position where the load was set to zero. Subsequently, moments of 1 Nm were gradually applied in flexion/extension (FE), right and left lateral bending (LB), and right and left axial rotation (AR) with a rotation speed of 1°/s. During the experiments, the spines were protected from drying by spraying intermittently with 0.9% saline. Specimens were tested for three continuous cycles, and the data from the 3rd cycle were analyzed using a custom program written in Matlab (Mathworks, Natick, MA). Movement in all three directions was plotted as a function of

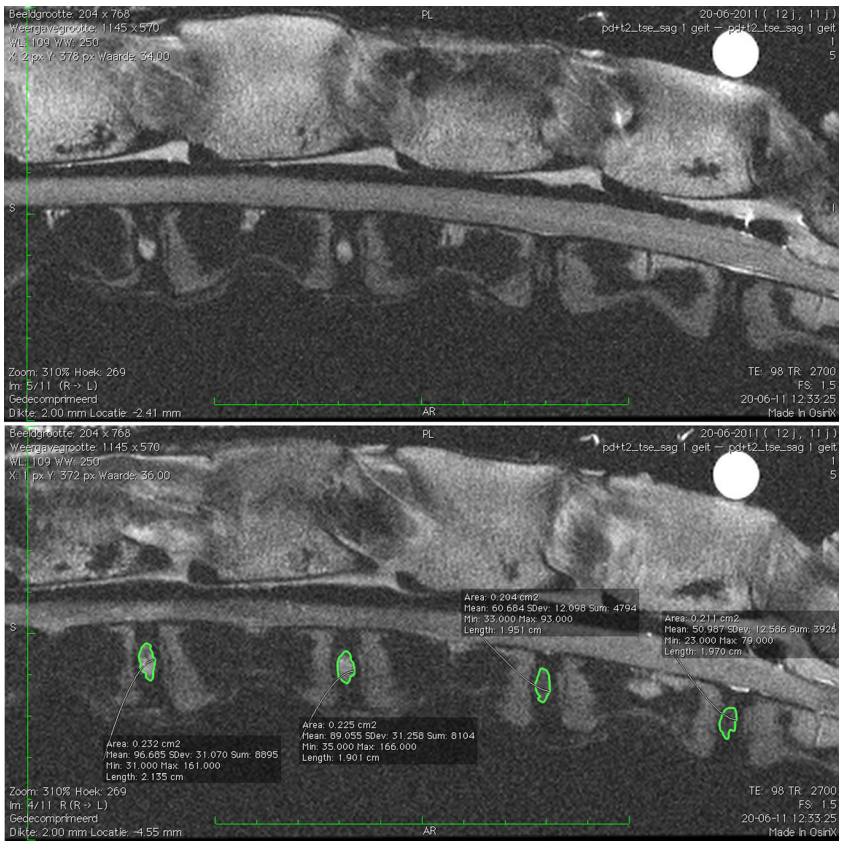

Figure 1. Images from a representative part of an MRI scan showing two control IVDs (left) and two injected IVDs (right). On the second image, MRI index lines are drawn.

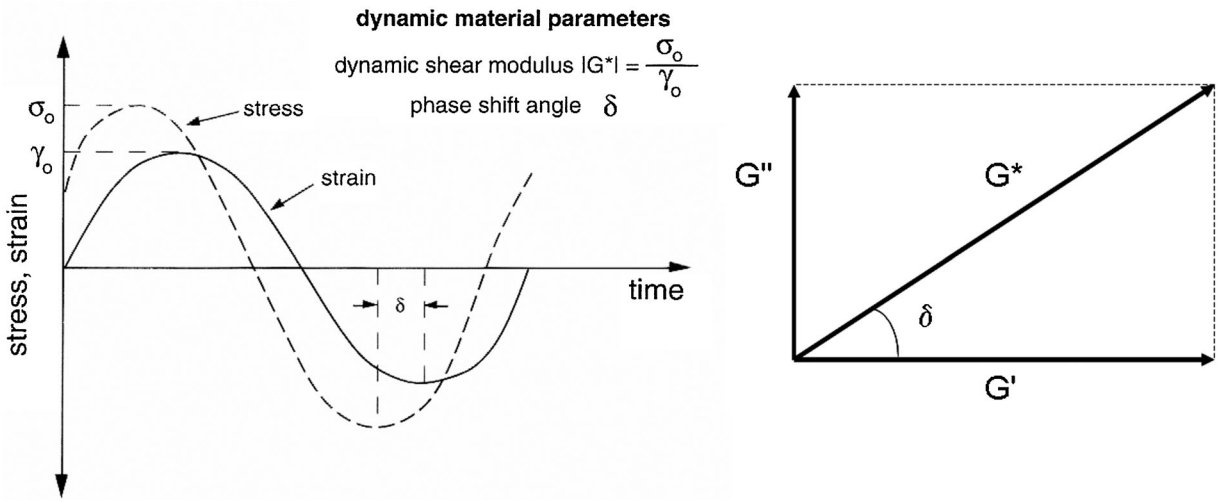

Figure 2. Schematic showing the associations between various rheological parameters: complex shear modulus (G^*), elastic (G'), and viscous modulus (G''), phase shift angle (δ), shear stress (σ), and strain amplitude (γ).

load to obtain load–deflection curves. The range of motion (ROM) of each segment was calculated from these curves as the motion between +1 and −1 Nm. The neutral zone stiffness (NZS) was calculated as the slope of the curve in the NZ after fitting a summed sigmoid function, as described previously.[27] When the neutral zone could not be defined, the stiffness was derived from the slope around 0 Nm (−0.5 to +0.5 Nm).

Rheological Characterization

The motion segments were stored at −20°C until the day of rheological testing. After thawing, IVDs were cut from the endplates with a surgical knife, and NPs were isolated using a 9 mm circular trephine. Rheological testing was performed as described previosuly[21] on a stress-controlled rheometer (Paar Physica MCR501; Anton Paar, Graz, Austria) in parallel plate configuration (20 mm diameter, 2–3 mm gap). To ensure good contact with both plates and prevent sample slipping, sandpaper was attached to the plates. Also, a compressive strain of 10% of initial NP height was applied, temporarily increasing the normal force to ~0.12 N. The tests were performed at 37°C in a humidified chamber. To exclude any time-dependent viscoelastic behavior, samples were first equilibrated for 5 min at 0.5 Hz applying 0.5% strain. Meanwhile, normal force returned to values below 0.03 N in all samples. Next, we performed frequency sweep measurements over an angular frequency range of 0.01–30 Hz at fixed strain amplitude of 0.5%. The shear modulus G^* follows from the ratio between shear stress (σ) and strain amplitude (γ). G^* is a complex quantity with an elastic storage modulus (G') and a viscous loss modulus (G''). The ratio between G'' and G', indicating energy dissipation, is called the damping factor and equals the tangent of the phase shift angle ($\tan\delta$) between shear stress and strain (Fig. 2). As the sample diameters were smaller than the plates, the measured values were corrected before further evaluation using the same correction factor applied earlier.[21] Values for G^*, G', G'', and $\tan\delta$ were taken from the frequency sweep at 1 Hz (2π rad/s). Finally, the stiffness of NP samples in (unconfined) compression was tested under static loading conditions. This was performed by applying compressive strain at 0.01 mm/s until a normal force of 50 N was reached. Data from compression testing were analyzed as described previously.[28] Briefly, a compressive modulus at low strain was calculated by linear regression as the slope of the stress–strain curve from ~10% to 30% strain. Compressive strain was calculated as $1 - H/H_0$ (H = height and H_0 = initial height), and stress was calculated as force divided by area.

Water Content

All samples were weighed after rheological testing and after freeze-drying (speedvac). Water content was defined as the difference between wet and dry weight, expressed as a percentage of wet weight.

Statistical Analysis

To analyze the differences between CABC-injected and control IVDs, independent Student's t-tests were used on the disc height and MRI indices. To compare outcome parameters from mechanical and viscoelastic experiments, a General Linear Model was used with goat number as random factor. The analyses were performed using SPSS Statistics version 17.0 (SPSS, Inc., Chicago, IL), where $p < 0.05$ was considered significant.

RESULTS

All goats showed normal ambulatory activities on the 1st post-operative day. One goat developed a superficial wound infection 4 weeks after surgery. Treatment consisted of subcutaneous antibiotics (penicillin–streptomycin) during 5 days and daily rinsing. The wound healed 6 weeks postoperatively, and the animal did not show signs of illness, discomfort, or pain during that period. Eventually, the superficial wound infection was considered not to be of influence as this goat did not appear as an outlier in any of the outcome parameters. Otherwise, goats appeared healthy, and body weight was either maintained or increased during follow-up. At autopsy, no abnormalities were found at the site of surgery or elsewhere. Macroscopically, no major indications of degenerative disc disease (e.g., osteophytes, endplate fractures) were identified.

Radiology

DHI of the CABC-injected IVDs decreased significantly ($p < 0.01$) by 6% compared to control levels (Fig. 3). No endplate fractures or osteophytes were observed in any of the lumbar spines on radiological analysis. The MRI index of CABC-injected IVDs was significantly lower than controls, with an average difference of 22% ($p < 0.001$; Fig. 3). On MRI, no gross aberrations were observed on the lumbar spine. All cartilaginous endplates were intact, and no annular tears were seen.

Biomechanical Testing

Load–deflection curves were plotted for each tested IVD ($n = 32$) in all three directions: FE, LB, and AR. In 9 of 96 curves, the NZ had to be indicated manually because the maxima in the 2nd derivative were not adequately identified. In nine other plots, no maxima in the 2nd derivative (due to lack of a double sigmoid function) were identified, and the NZ stiffness was calculated as the slope of the curve around 0 Nm. In two curves fitting was impossible, hence the curve resulting from the downward movement of cycle 3 was used to calculate NZS. In LB and AR, the NZS decreased significantly with mild degeneration by 31% and 27%, respectively. ROM increased by 23% ($p < 0.001$) for LB and by 32% ($p < 0.01$) in AR. No significant differences were observed in flexion/extension after CABC-injection (Fig. 4).

Rheometry

In all samples, the elastic storage modulus G' was three to four times larger than the viscous loss modulus G'' indicating a more solid than liquid behavior of the NP. This is also reflected by the damping factor tan δ that remained at 0.30 ± 0.02 with no significant difference between the CABC-injected and control NPs. The variation in the complex shear modulus G^* between goats was remarkably large (Fig. 5). In spite of this, a significant ($p < 0.05$) decrease of ~10% in both elastic and viscous moduli was observed in mildly degenerated NPs within goats (Fig. 6). One goat stood out of this tendency and showed a non-significant increase in the rheological parameters. The mean compressive modulus was 0.46 ± 0.17 kPa. No significant difference was observed in this parameter between CABC-injected and control NPs.

Water Content

The relative water content of the NPs was not different between CABC-injected ($74.9 \pm 1.4\%$) and healthy samples ($74.7 \pm 1.1\%$). Furthermore, water content was not correlated to any of the rheological parameters.

DISCUSSION

We evaluated the biomechanical characteristics of SMSs and viscoelastic properties of the NP in healthy and mildly degenerated goat IVDs. Imaging techniques were applied to assess a decrease in disc height and a lower signal intensity of the NP on MRI. These findings confirmed the presence of mild degeneration after CABC injection into the IVDs of the large animal model as described previously.[22,23] Moreover, we observed a decrease in NZ stiffness and an increase in ROM after inducing mild disc degeneration. This further corroborates our disc degeneration model, as mild degeneration is generally correlated with increased IVD mobility in previous biomechanical research on both animal[29,30] and human spines.[31–35]

Rheological tests were performed on isolated NPs, resulting in elastic (storage) and viscous (loss) moduli in the same range as both human and goat values from previous studies.[21,36] However, we found a remarkably large inter-animal variation in viscoelastic moduli. As the measured values are far more consistent within each goat, this suggests that the large differences are due to varying animal characteristics,

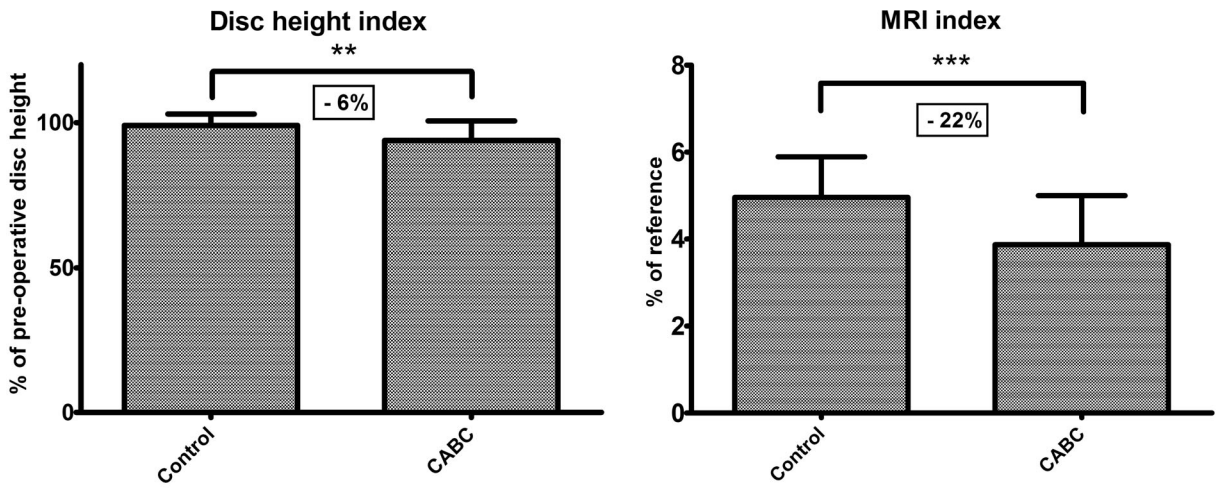

Figure 3. Disc height and MRI index of control IVDs ($n = 24$) and levels injected with CABC ($n = 24$). Data plotted as mean and SD [$^{**}p < 0.01$; $^{***}p < 0.001$].

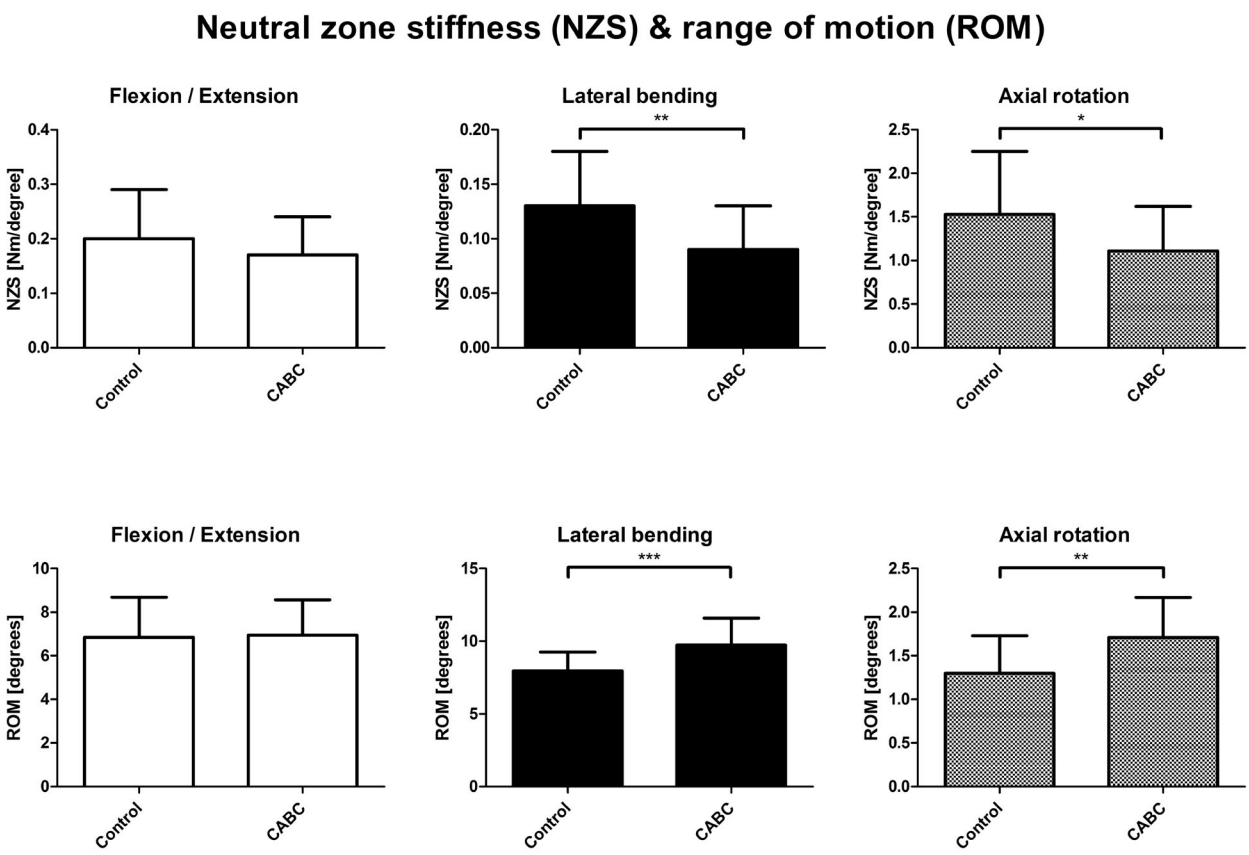

Figure 4. Neutral zone stiffness and range of motion in flexion/extension, lateral bending, and axial rotation for CABC-injected and control intervertebral discs. Data plotted as mean and SD [$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$].

rather than an artifact of sensitive measurement methods. Moreover, in a comparable study on cadaveric human NP material, an even larger range of values for the moduli was observed.[36] Whether inter-individual environmental differences, congenital properties, or specific circumstances during (embryological) development can account for this wide variation among subjects remains to be elucidated.

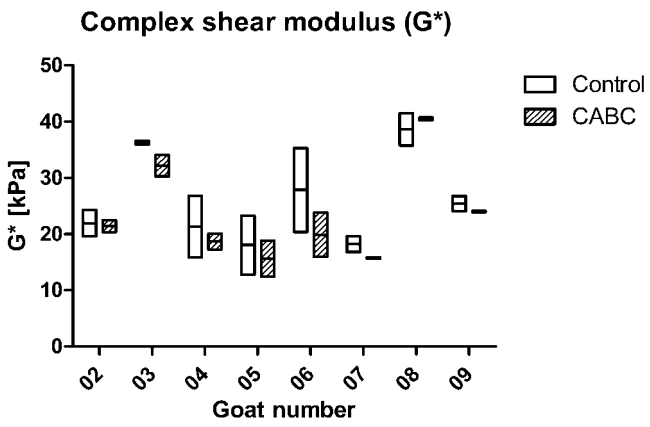

Figure 5. Inter-animal variation in the shear modulus (G^{*}) and difference between control and CABC nucleus pulposus. Each box represents two IVDs ($n = 2$, in total $n = 32$).

In spite of the relatively large inter-animal differences, we observed a modest but significant decrease in viscoelastic moduli (~10%) after mild degeneration. Interestingly, this is in contrast to an earlier study on human cadaveric material,[37] where shear moduli increased with age and degeneration. In our model, goat discs are mildly degenerated (Thompson grade II–III)[23] whereas Iatridis et al. reported degeneration up to Thompson grade V. In addition, degeneration in human IVDs develops over many years, while induction of the mild degeneration in this study was achieved within 12 weeks. This is a relatively short period for extracellular matrix changes—like collagen type I deposition—to develop, resulting in viscoelastic changes. Possibly, SMSs lose stiffness at an early degenerative stage, due to a slight decrease in NP viscoelastic moduli, while after a longer period degeneration is characterized by an increase in elastic modulus.

In our study, the damping factor tan δ (ratio between the viscous and elastic moduli), indicative of energy dissipation, remained unchanged with mild degeneration. Again, this differs from human cadaveric studies,[37] where a decrease in energy dissipation with increasing degeneration was observed. Thus, the viscoelastic behavior of degenerated human NPs shifted from "fluid-like" to more "solid-like," whereas our goat NPs did not show this transition. This

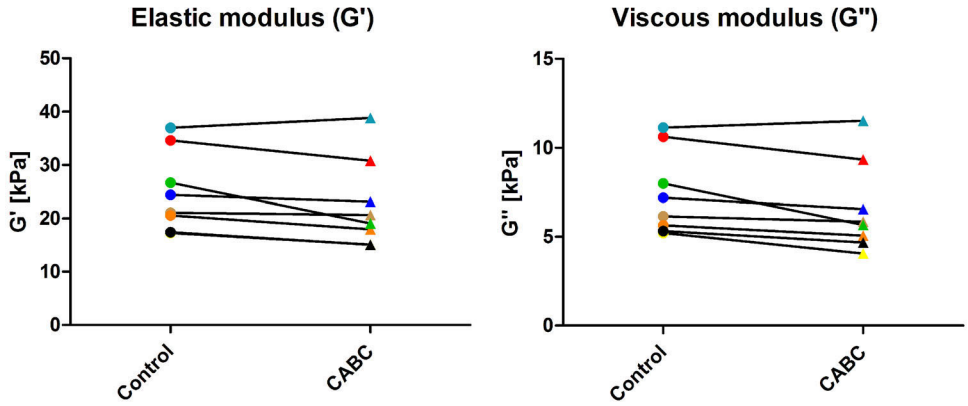

Figure 6. Elastic storage (G') and viscous loss modulus (G'') of CABC-injected NPs compared to healthy control levels. A slight decrease ($p < 0.05$) in both the elastic and viscous modulus was observed after analysis with a General Linear Model ($n = 2$ per group, in total $n = 32$).

contrast might be explained by the difference in duration of the degenerative processes. Eventually, this shift could also arise in the goat model by the formation of fibrous tissue. After all, our model has previously demonstrated increased gene expression of collagen type 1 in severely degenerated discs.[38] The unaltered energy dissipation is also in accordance with the unchanged water content between healthy and degenerated NPs.

Recently, other researchers also found unaltered water content in chemically degenerated rat IVDs[39] confirmed in goat IVDs by unpublished data from our group. Both quantified the water content of the NP, determined by weighing before and after dehydration. Chemically induced degeneration is characterized by a loss of glycosaminoglycans (GAG) from the NP due to cleavage by CABC, injected into the IVDs. Previous studies documented this proteoglycan loss as a decreased GAG/collagen ratio (30:1–15:1)[38] and as a reduced GAG content in the NP (43% decrease).[30] As the declining GAG content is followed by diffusion of water out of the NP, water loss will be proportional to the lost amount of proteoglycan. As a consequence, (relative) water content remains unchanged. In more advanced stages of degeneration, the increase of collagen type I in the NP might increase the protein content, explaining the observation of loss of water content and increased elastic modulus by others.[37]

Injection of a 0.25 U/ml CABC solution into goat IVDs results in a relatively mild degeneration compared to the severity of the degenerative process in other (human) studies. Our model was deliberately designed to mimic a mild degeneration, as we consider this process to be reversible and thus suitable for potential regenerative treatment. The drawback of inducing more severe degeneration is the potential extra damage to endplate or annulus fibrosus (AF), rendering regenerative strategies less opportune. Nevertheless, this mild degeneration is sufficient to produce significantly altered imaging, histological, biochemical, and mechanical outcomes. Hence, our model represents a valid method for investigating mild IVD degeneration and subsequent testing of evolving regenerative therapies for NP repair.

Unlike the relatively small decrease in viscoelasticity, segmental biomechanical characteristics changed consistently (~30% decrease in NZ stiffness and increase in ROM) with mild degeneration. Consequently, the impact of viscoelastic properties on the biomechanical behavior of the whole SMS is marginal. We suggest that hydrostatic pressure in the NP influences biomechanical behavior of the segments more than NP viscoelasticity. Loss of hydrostatic pressure was identified before as a cause of biomechanical changes.[40] Biochemical and mechanical stimuli are important for the direction of regenerative cells in IVD matrix tissue. Therefore, when developing regenerative therapies, emphasis might be shifted towards the mechanobiological interaction of scaffolds with regenerative cells, instead of mimicking native NP material properties.

In conclusion, we established mild degeneration in lumbar IVDs in a goat model as confirmed by radiology and biomechanical testing. We observed a slight decrease in both elastic and viscous moduli, but the ratio between these remained unchanged, indicating that the NPs did not become more fluid-like or elastic-like. This is consistent with our measurements on the water content of the NPs, which also remained unchanged. The large variability between animals in viscoelasticity, but not in segmental biomechanical characteristics, implies that the influence of viscoelasticity within the NP on the mechanical behavior of the whole SMS is marginal. Therefore, when developing NP replacement materials, the focus should be on other design criteria for NP regeneration, like biochemical cues and swelling pressure.

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